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14. ABSTRACT
Neurofibromatosis type 1 (NF1) is a common single gene neurogenetic disorder characterized by tumors in peripheral nerve terminals. A large fraction of patients also have learning problem. Such learning phenotypes have been recapitulated in animal models, including in mouse and Drosophila mutants. This proposal mainly examines functions of the neurofibromatosis type 1 (NF1) gene and its regulated signal transduction pathways in learning and memory in Drosophila. We have reported in previous annual report that the NF1 C-terminal mediating Gsα/NF1-dependent activation of adenylyl cyclase (AC) and the GAP-related domain (GRD) for regulating Ras activity, such as Ras/NF1-dependent AC activation. Over last funding period, we mainly focused on studying roles of these two distinct functional domains in learning and memory. Our study revealed that both immediate memory and long-term memory (LTM) are abnormal in NF1 mutants. Our analysis of effects of clinically relevant mutant NF1 genes concluded that LTM formation only requires GAP function of NF1 and is mediate by the GRD while immediate memory only involves the C-terminal. Thus, NF1 is required for formation of two memory components but through distinct functional domains that regulates different signal transduction pathways. Moreover, a spatial and temporal analysis revealed a role of NF1 in learning and LTM in distinct brain regions and at the LTM retrieval, but not in memory formation or consolidation.

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Introduction

The major hypothesis of this proposal is of that distinct regions of the NF1 protein control both Ras/NF1 and Gsα/NF1 stimulated adenylyl cyclase (AC) activity, and that these regions can be readily identified by examining the effect of mutated human genes on the phenotype of *Drosophila* NF1 null mutants. Specifically, we proposed that Gsα/NF1 activated AC pathway mediates learning or immediate memory but exerts no effect on long-term memory (LTM) while the Ras/NF1 activity is crucial in formation of LTM.

In last four funding years, we have been able to identify two functional domains in NF1 through biochemical assays and body size measurement: The C-terminal mediates Gsα/NF1-dependent AC activation and the GRD for Ras/NF1-dependent AC activation (Hannan et al., 2006). Further behavioral analysis revealed that the C-terminal was only involved in learning while the GRD-regulated Ras activity is essential for LTM formation (Ho et al., 2007). Our preliminary data indicated that NF1 had no effect on LTM formation but specifically required for LTM retrieval. This last part of work has not been published and further confirmation may be needed.

In addition to specific studies of NF1-related cognitive effects, we also began to investigate how mutations identified in Noonan syndrome (NS) affect LTM. Noonan syndrome is one of so-called Ras-related disorders as NF1 is. It shares symptoms with NF1 and is also resulted from excessive activation of Ras as is in case of NF1 (ref). We hope that such studies will help understanding of pathogenic mechanisms underlying NF1. We showed that the clinically relevant NS mutations also disrupt LTM in Drosophila. This work has resolved in a publication (Cell, in press).

Body

We have reported that NF1 mutations disrupt learning or immediate memory through alteration of the cAMP pathway (Guo et al., 2000). However, the learning defect reported in mouse NF1 knockouts is linked to the elevated Ras activity (Costa et al., 2002). To reconcile the difference, we have examined other components of memory in *Drosophila*. We found that in addition to immediate memory, LTM was also defective. Thus, we focused on dissecting molecular bases of NF1's role in both immediate memory and LTM.

For the purpose of dissection, we generated various mutant human NF1 (hNF1) genes with mutations identified clinically in patients (Hannan et al., 2006). Transgenic flies have been made to carry the normal and mutant hNF1 transgenes in a null NF1 genetic background. We also made transgenic flies that carry various deletions, including the C-terminal and GRD regions. With these transgenic flies, we identified two independent NF1 functional domains and determined their respective roles in learning and memory.

1. Identification of two independent functional domains.

Although the functions of NF1-GRD have been extensively characterized in both vertebrates and flies, the NF1 region that is responsible for mediating AC activation has yet to be identified even though this pathway has been shown to be critical in mediating neurotransmission in both vertebrates and flies, in controlling development of body size, in mediating learning in Drosophila (Guo et al., 1997, 2000; The et al., 1997; Tong et al., 2002; Dasgupta et al., 2003). For this purpose, we first examined effects of various deletions to

localize the region that could affect body size and AC activation. We developed a preparation that allowed us to assay activation of AC via application of a range of neurotransmitters, neuromodulaters, and growth factors. We found that both body size and Gsα/NF1 dependent AC activation were not affected by the NF1-GRD or mutations that affect Ras activity as well as N- terminals, but were affected by the C-terminal fragment. This part of NF1 is necessary and sufficient in mediating Gsα/NF1 dependent AC activation. This represents the first demonstration for functions of an NF1 region that is outside of the GRD and that its function is independent of the GRD. In addition, all the results have been presented in a recent publication in the journal of Human Molecular Genetics (Hanna et al., 2006).

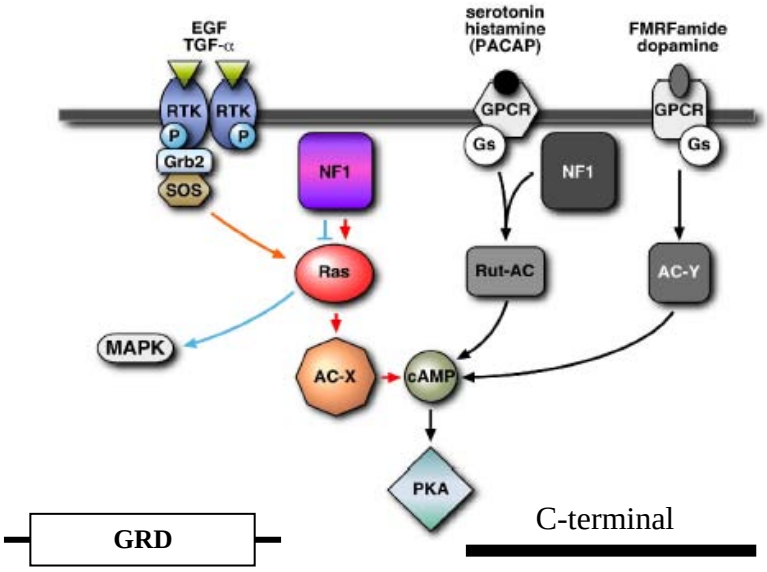

Fig. 1. In *Drosophila* olfactory related memory consists of several distinct components, acquisition (or immediate memory), short-term memory, mid-term memory, anesthesia-resistant memory and LTM (Tully et al., 1994). These components can be isolated through different training paradigms or through genetic and pharmacological manipulations. In our study, we have focused on immediate memory and LTM. Immediate memory is assayed immediately after one cycle of training while LTM is elicited through 10 repetitive training trails with 15 min of resting interval between trails (Tully et al., 1994). The LTM score is determined usually 24 hours after training.

2. NF1 in Learning and Memory Retrieval

In previous studies, we have generated various mutant human NF1 (hNF1) genes with mutations identified clinically in patients (Hannan et al., 2006). Transgenic flies have been made to carry the normal and mutant hNF1 transgenes in a null NF1 genetic background. We also made transgenic flies that carry various deletions, including the C-terminal and GRD regions. Thus, we could assay how immediate memory and LTM were affected by these mutations and deletions.

All these studies allowed us to show that LTM formation only requires GAP function of NF1 and is mediate by GRD. In other words, LTM was abolished by mutations in the GAP domain that either diminish GAP activity or prevent its binding to Ras. LTM formation was also blocked by deletion of the GRD. In contrast, these mutations or deletions had no effects on immediate memory. Immediate memory was altered only by the deletion of the C-terminal.

In our recent studies we have examined in which regions of the brain NF1 is required for LTM.
Learning and memory in the fly brain has been mostly attributed to the antennal lobes (AL) the mushroom bodies (MB) and the central complex (CC) (Liu et al., 2006; Yu et al., 2006; Popov et al., 2003; Davis, 1996; Wu et al., 2007). We performed experiments of rescue of learning or LTM in NF1 mutants. In these

experiments we used Gal4 lines to target the expression of hNF1 in MB and CC in flies with *Nf1*-null mutant background. All three MB-Gal4 lines, c747, 201Y and OK107 significantly rescued the learning defect when used to express hNF1. On the other hand, the CC-Gal4 lines, c107 and Feb170, are not capable to rescue the learning defect in NF1 mutants. These transgenic flies were also subjected to spaced training and tested for their performance in LTM. All MB lines partially rescued the 24-hour memory defect compared to the control group. Interestingly, the CC-Gal4 lines also partially rescued 24-hour memory after spaced training. These data suggest that NF1 is required to function in both the MB and central complex to mediate LTM, while only in the MB to mediate learning.

In more recent studies we started to examine the relation between learning and LTM deficit and the temporal requirement of NF1. We showed that learning and LTM depend on the C-ter region and GRD domain of NF1, respectively (Ho et al., 2007). However, how the defects in learning affect LTM?
We found that the acquisition phase to form LTM is not affected in absence of NF1, which seems to be contrary to our previous finding of the requirement of NF1 in learning (Guo et al., 1997; Ho et al., 2007). However, the classical experiment of learning involved 1 cycle of training, while 10 spaced cycles are used to form and examine LTM (usually 24 and 48 hr memory). Strikingly, Nf1-null mutant flies tested immediately after 10 cycles of spaced training showed normal memory retention, supporting a normal learning after a longer trial of acquisition. This experiment indicates that the LTM defect in Nf1-null mutants is not resulting as a consequence of a poor memory acquisition. Thus, these experiments showed that the GRD domain disrupt LTM independently of the acquisition phase.

To understand if NF1 participates in consolidation, storage and/or retrieval we used acute expression, in adult animals, of a wild type *Nf1* transgene in the null mutant background. The heat shock-controlled expression of Nf1 (hsNF1;$Nf1^{P2}$) restores the H-Ras-stimulated AC activation to wild type levels, as well as LTM (Ho et al., 2007; Hanna et al., 2006). However, in those experiments the 2-hr heat shock used did not provide the temporal resolution to differentiate if NF1 is needed in consolidation, storage or retrieval. Nevertheless, a 30 min heat shock, 2 hrs before memory test revealed a role of NF1 in retrieval (Fig. 2 C and D), but not in memory formation or consolidation (Fig. 2 A and B).

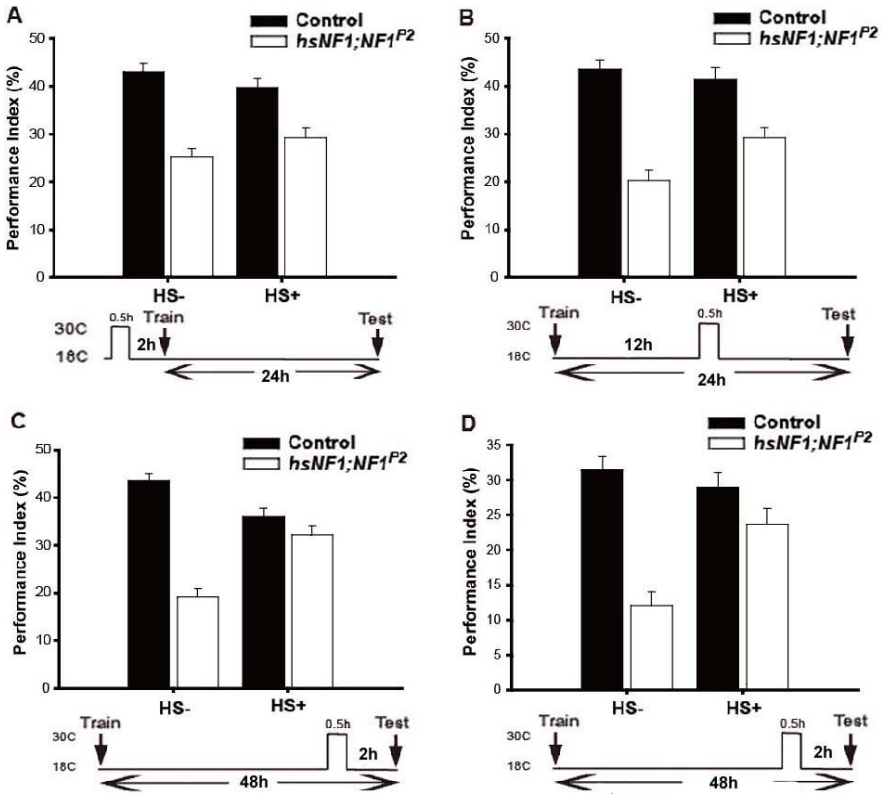

Fig. 2. NF1 is required in retrieval of LTM. NF1 null mutant flies carrying an NF1 transgene under control of a heat shock promoter (hsNF1;$NF1^{P2}$) and control flies (K33) were developed at 18 °C. Acute expression of a wild type *Nf1* transgene in the null mutant background was controlled by a 30 min heat shock at 30 °C (HS+) 2 hr before training (**A**), 12 hrs after training (**B**) or 2 hrs before test at 24 (**C**) or 48 hrs (**D**) after training. PI = mean ± s.e.m., n = 8; p < 0.001.

3. Noonan Syndrome

Elevated MAPK activation is a consistent biochemical hallmark of Noonan syndrome (NS) as well as of other phenotypically related Ras/MAPK disorders, such as NF1, Nuerofibromatosis-NS; cardio-facio-cutaneous and Costello syndromes (for review, see Gelb and Tartaglia, 2006; Aoki et al., 2008). Nf1 and PTPN11 are the major genes causing NF1 and NS, respectively. Both, NF1 and NS, are related and genetically heterogeneous disorders characterized by a number of developmental defects. Strikingly, in both disorders, affected individuals present malignancies and cognitive alterations. These common features may be based in that Nf1 and PTPN11 encode a negative and positive regulator of the Ras pathway, respectively.

To gain insights into the pathogenesis of the cognitive defects, we investigated the behavioral effects of clinically relevant PTPN11 gain-of-function (GOF) mutations in Drosophila. The orthologous gene in fruit flies is corkscrew (csw), which encodes a protein tyrosine phosphatase that is structurally and functionally conserved with SHP-2 (Freeman et al., 1992; Perkins et al., 1996; Neel et al., 2003).
Our study reveals that NS mutations, expressed in MB can disrupt LTM, but not immediate memory (Fig. 3) (Pagani et al., 2009). Moreover, the duration of resting intervals required for inducing LTM can be regulated by activity levels of the protein tyrosine phosphatase corkscrew (CSW) in Drosophila and altered by gain-of-function mutations associated with Noonan syndrome. Our studies also showed that wild-type CSW overexpression dramatically shortens the inter-trial interval required for LTM induction. We demonstrate that, even when expressed only in adulthood, GOF-CSW impairs LTM formation, which can be rescued by lengthening the inter-trial interval. We also document that LTM-inducing training regimens generate repetitive waves of mitogen-activated protein kinase activation, providing a rationale for the varied abilities of our Drosophila models. Thus, we establish that the spacing effect has a molecular basis and CSW/SHP-2 is key in its regulation (Pagani et al., 2009).

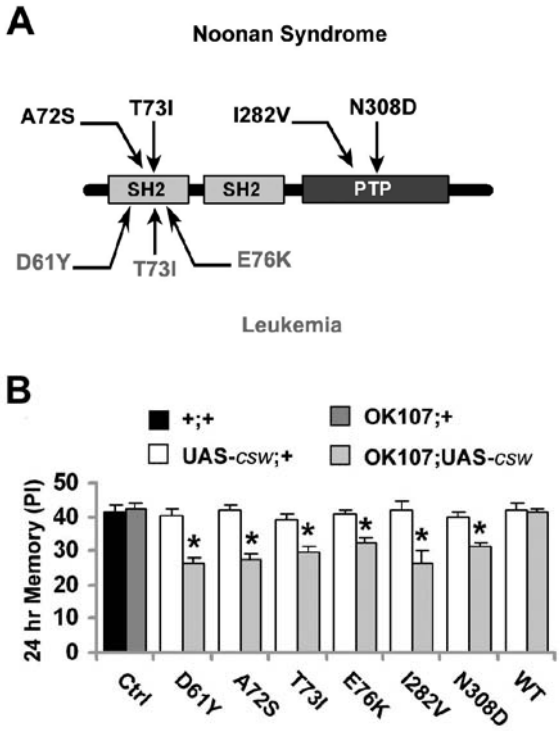

Fig. 3. Noonan syndrome mutation Impairs 24-Hour Memory.
(A) Schematic representation of the CSW protein mapping point mutations detected in Noonan syndrome (top) and in leukemia (bottom). (B) 24-hr memory after spaced training in fly lines only carrying the transgene (UAS-csw;+) or expressing csw transgenes (OK107;UAS-csw). Transgene expression of csw mutant (from D61Y to N308D) and csw wild-type (WT) was targeted to the mushroom body using OK107-GAL4 (OK107;UAS-csw). Memory was reduced in all fly lines expressing mutants alleles (OK107;UAS-csw) compared with control (+/+ or OK107;+) flies, but not in lines only carrying those csw transgenes (UAS-csw;+). Bars, mean + SEM (n=8). Asterisks indicate $p<0.05$.

Key Research Accomplishments

We have shown that NF1 is necessary for two memory components through distinct and functionally independent domains. The C-terminal is required for immediate memory while the GRD or GAP activity is essential for LTM. Strikingly, the information acquisition for immediate memory appear to be independent of the acquisition for LTM in a way that the well known learning disability in NF1 null mutants, only associated with MB, can be prevented by additional sessions of learning without effects on the poor LTM. However, for LTM, NF1 is required in two different brain regions (i.e. MB and CC) for memory recall, but not for memory formation as it was thought. Importantly, NF1 and CSW, even when it is expected to be acting through Ras/Erk pathway, they are involved in LTM retrieval and LTM formation respectively.

Reportable Outcomes

The effort has resulted in the identification of two NF1 domain (C-term and GRD) acting in different pathway (Hannan et al., 2006) and involved in immediate and LTM, respectively (Ho et al., 2007).
On the other side, whereas NF1 and CSW can regulate the Ras pathway, they appear to be involved, respectively, in memory retrieval (manuscript in preparation) and in LTM formation (Pagani et al., 2009)

Conclusion

First, through analysis of the effect of mutations and distinct segments of nf1 on the activation of AC and MAPK, we discovered that NF1 was involved in two distinct signaling pathways. Second,
through assaying effects of immediate memory and long-term memory of hNF1 mutants in *Drosophila*, we have revealed that the C-terminal of NF1, which regulates Gsα/NF1 dependent AC activation, is involved in immediate memory and the GAP-related domain, which regulates Ras activity, is required for LTM retrieval. Thus NF1 is involved in two memory processes, but through different signal transduction mechanisms and brain regions. Third, by analyzing the effects of GOF CSW on LTM, we discovered that a defect in the spacing effect for LTM formation provide a mechanistic basis for the LTM defect in a model of Noonan syndrome.

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